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Complement Biomarkers as Predictors of Disease Progression in Alzheimer's Disease

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Abstract. There is a critical unmet need for reliable markers of disease and disease course in mild cognitive impairment (MCI) and early Alzheimer's disease (AD). The growing appreciation of the importance of inflammation in early AD has focussed attention on inflammatory biomarkers in cerebrospinal fluid or plasma; however, non-specific inflammation markers have disappointed to date. We have adopted a targeted approach, centered on an inflammatory pathway already implicated in the disease. Complement, a core system in innate immune defense and potent driver of inflammation, has been implicated in pathogenesis of AD based on a confluence of genetic, histochemical, and model data. Numerous studies have suggested that measurement of individual complement proteins or activation products in cerebrospinal fluid or plasma is useful in diagnosis, prediction, or stratification, but few have been replicated. Here we apply a novel multiplex assay to measure five complement proteins and four activation products in plasma from donors with MCI, AD, and controls. Only one complement analyte, clusterin, differed significantly between control and AD plasma (controls, 295 mg/l; AD, 388 mg/l: $p < 10^{-5}$). A model combining clusterin with relevant co-variables was highly predictive of disease. Three analytes (clusterin, factor I, terminal complement complex) were significantly different between MCI individuals who had converted to dementia one year later compared to non-converters; a model combining these three analytes with informative co-variables was highly predictive of conversion. The data confirm the relevance of complement biomarkers in MCI and AD and build the case for using multi-parameter models for disease prediction and stratification.

Keywords: Alzheimer's disease, biomarker, complement, inflammation

INTRODUCTION

The current lack of plasma biomarkers for diagnosis, stratification, or prediction of outcome in AD is a major deficit that compromises early diagnosis and patient selection for trials of novel therapies [1–3]. In particular, biomarkers that aid early diagnosis and/or predict progression from MCI to AD are a critical need. Primarily of value in the near future to aid in the recruitment to secondary prevention trials, such markers predictive of progression in prodromal states might become of clinical value in future as disease modification therapies become available. A few plasma markers have been described but are untested in preclinical disease and likely unsuitable for early diagnosis [3]. The goal for current studies is

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to deliver a highly informative plasma biomarker or set of markers that enable early diagnosis and predict disease course [4, 5]. The recognition that inflammation is an important player in AD and likely an early event in disease pathogenesis brings to the fore the potential use of markers of inflammation [6]. Non-specific indicators of peripheral inflammation such as C-reactive protein and inflammatory cytokines have proved unreliable as markers of disease or disease progression [7, 8], suggesting that a more targeted approach, focusing on specific inflammatory pathways might be more rewarding.

Complement, a pillar of innate immunity and key player in driving inflammatory responses to injury and infection, is a prime target pathway, implicated in the pathogenesis of AD through genetic, pathological, and animal model evidence [9–11]. Several published studies have explored whether plasma levels of complement components, regulators, or activation products are altered in AD or predict progression in the disease. In one of the first untargeted proteomics analyses of plasma in AD, complement factor H (FH) was found to be elevated in AD plasma compared to controls [1]; an intriguing observation given the genetic association between FH and age-related macular degeneration (AMD), another amyloid related condition [12, 13]. However, one other targeted study using a different methodology did not find association of FH with AD [14], and the common polymorphism in FH (Y402H) that is a strong risk for AMD does not significantly impact AD risk when assessed at the genetic level [15, 16]. Levels of plasma clusterin, a modulator of the terminal complement pathway, have been associated with disease, disease subtype, and rate of progression in several studies [17–20], although as with FH, negative findings have also been reported [21]. Plasma factor I (FI), measured semi-quantitatively, was highly predictive of brain atrophy in AD [22]. C1s and C9 have been implicated at the genetic level in pathway analysis studies [23].

We used these published findings, together with relevant genetic data, to identify a candidate complement biomarker set. Here we describe the use of a custom-made ten-analyte multiplex set on the MSD platform to measure selected candidate AD biomarker complement proteins and activation products. The set comprised FH (measured as the individual Y402 and H402 alleles [22]), clusterin, FI, C1s, C9, C4d, Bb, iC3b, and TCC. The four complement activation products selected for measurement included markers of classical (C4d, iC3b), alternative (Bb, iC3b), and terminal (terminal complement complex; TCC) pathway activation. The study comprised two arms, one in which AD samples were compared to matched controls, and the other in which enrolment samples from individuals with MCI who had subsequently converted to AD when re-assessed 12 months later (convertors) or who had remained stable over the period of assessment (non-convertors) were compared. Of the analytes measured, only clusterin differed significantly between matched controls and AD patients, while three analytes, clusterin, FI, and TCC, differed significantly between MCI convertors and non-convertors. For each study arm, models were built comprising the analytes that differed significantly together with relevant co-variables (APOE status, age). Each of the models was highly predictive with overall predictive power (from area under the curve [AUC] in receiver-operating characteristic [ROC] analysis) of 0.78 for AD versus control and 0.85 for MCI convertor versus non-convertor.

The findings further implicate complement as a contributor to disease progression in AD and make the case for building multi-parameter models including informative complement biomarkers, non-complement biomarkers and other patient data that enable patient stratification and prediction of progression.

MATERIALS AND METHODS

Samples

All samples were first visit samples obtained from the previously reported AddNeuroMed and Dementia Case Register studies [24, 25]. For comparison of AD and control groups, a total of 292 first visit samples (106 AD, 186 controls) were selected. The mean age for the AD samples was 74.7 years and mean age of controls was 78.1 years. The sample set was randomly divided into a training set comprising 206 samples (75 AD, 131 controls) to generate the model, and a testing set comprising 86 samples (31 AD, 55 controls) to assess the accuracy of the model. In a separate analysis, 189 samples obtained from patients diagnosed with MCI at the point of sampling were tested. Note that all measurements here were on this first sample when all were classified as MCI. Upon re-assessment 12 months later, 49 of these patients had converted to AD while 140 had not converted. The mean age at first presentation was 75.2 years for the convertors and 76.3 years for non-convertors. These 189 MCI samples were randomly divided into a training set of

133 (98 not converted, 35 converted), and testing set of 56 (42 not converted, 14 converted) for analysis.

Assay development and multiplexing

Ten complement analytes were selected for this study, six components or regulators (C1s, C9, clusterin, FI, FH-Y402, FH-H402), and four activation products (iC3b, C4d, Bb and TCC). Analyte choice was informed by reference to previous studies of complement biomarkers in AD, and availability of reagents; the activation marker set was chosen to interrogate classical (C4d, iC3b), alternative (Bb, iC3b), and terminal (TCC) activation pathways. The FH-Y402 and FH-H402 allotypes were measured separately using highly specific monoclonal antibodies as described previously [26], and total FH concentration was obtained by summing the concentrations of the two allotypes. For each analyte, an antibody pair was selected from commercial or in-house sources (Table 1) and tested in ELISA for capacity to detect the analyte in plasma using purified proteins as standards. Selected antibody pairs were then tested in single-plex assays using high-bind plates from "ELISA Conversion Pack I" (MesoScale Discovery Platform [MSD], Rockville, Maryland, USA). Detection antibodies were conjugated to SULFO-TAG with ratio 1:12 according to the manufacturer's instructions. Single-plex assays were validated for reproducibility (intra- and inter-assay Coefficient of Variation [CV] <10%), sensitivity and dynamic range. For each analyte the range of plasma dilutions that enabled accurate quantitation was assessed; the optimal plasma dilution for measurement of all analytes in the set was then selected. Ten-plex plates (all analytes measured in a single well) were then printed by MSD using the supplied capture antibodies, and re-validated for reproducibility, sensitivity, and dynamic range and to confirm that all included analytes could be measured at a single plasma dilution. Ten-plex plates were also tested with mixtures of the analyte standards to ensure that there was no "cross-talk" between assays, an essential quality control in multiplex assays, and CVs for each analyte re-tested.

The assay protocol was as follows: Printed tenplex plates were blocked with 150 μl/well 3% BSA in PBS at 4°C overnight. Plasma samples were diluted 1:300 in assay buffer (PBS containing 1% BSA and 10 mM EDTA); 25 μl aliquots were then added in duplicate to wells. To calibrate the assays, a standard plasma was generated comprising a mixture of normal plasma and complement-activated plasma in which levels of all analytes were pre-calibrated against pure proteins using the single-plex assays. A calibration curve comprising a series of 5-fold dilutions of the standard plasma (1:5 to 1:6250) was run in duplicate on each plate. Two additional dilutions of standard plasma (1:250, 1:2500) in duplicate were used as inter-plate controls. Plates were incubated while shaking at room temperature for 60 min. After washing in PBS containing 0.01% Tween20, a mixture of the relevant SULFO-TAG-labelled detection antibodies diluted in assay buffer (1:100) was added and incubated as before. After washing, 150 μl of 2x reading buffer was added to each well and electrochemiluminescence (ECL) signal was immediately registered in a Sector S600 plate reader (MSD). ECL values in plasma samples were automatically converted to analyte concentration by reference to the calibration curve.

Statistical methods

All statistical analysis was conducted in R version 3.0.2. Correlation of individual analyte concentration with age at time of sampling was tested using Pearson correlation.

In both the AD:control comparison and the MCI convertor:non-convertor comparison, samples were split into training and testing sets as described above in order to reduce over-fitting of the model. Clustered mixed-effects linear modeling (using the lme4 and lmerTest R packages) was used to explore the associations between analyte concentration and disease status. Center of sampling was included as a random effects variable, and complement analyte, APOE-ε4 status (negative, heterozygous, homozygous), age at onset, and gender included as fixed effects variables. Variables that were found to be significant in the training set were retained in a refined model, which was tested for accuracy by applying to the test group. AUC was calculated, and ROC curves drawn to define the predictive power of the model.

RESULTS

Complement protein assays are sensitive and specific in multiplex formats

Each of the complement analyte assays translated from ELISA, through single-plex to multiplex without loss of performance as assessed by calculating CVs for each analyte. All analytes were accurately

Table 1
Antibody pairs for ELISA and multiplex. The table lists the antibody pairs used in the multiplex set and the sources of the antibodies. Quidel, https://www.quidel.com/; Hycult, http://www.hycultbiotech.com/; Comptech, http://www.complementtech.com/; Millipore, www.emdmillipore.com

Analyte/assay	Capture antibody (source)	Detection antibody (source)
C1s	MM Anti-C1s (M81, Hycult)	MM Anti-C1s (F33, in house)
C9	MM Anti-C9 (B7, in house)	MM Anti-C9 (6D4, in house)
Clusterin	RP Anti-Apolipoprotein J/Clusterin (AB825, Millipore)	MM Anti-Clusterin (MBI-40, in house)
FH-Y	MM Anti FH-Y402 (MBI-6, in house)	MM Anti-FH (OX-24)
FH-H	MM Anti FH-H402 (MBI-7, in house)	MM Anti-FH (OX-24)
FI	MM Anti-FI (7B5, in house)	RP Anti-FI (in house)
C4d	MM Anti-neo-C4d (A251, Quidel)	MM Anti-C4d (A213, Quidel)
TCC	MM Anti-neo-C9 (aE11, Hycult)	MM Anti-C8 (E2, in house)
iC3b	MM Anti-neo-iC3b (A209, Quidel)	MM Anti-C3b (C3-30, in house)
Bb	MM Anti-neo-Bb (A252, Quidel)	MM Anti-FB (JC1, in house)

MM, mouse monoclonal antibody; RP, rabbit affinity purified polyclonal antibody. Neo denotes neoepitope-specific antibody.

Table 2
Correlation between complement analyte concentration and age at time of sample. C9, FI, and TCC all showed a significant positive correlation with age in the populations sampled

Analyte	R^2	o
C1s	–0.016	0.82
C9	0.23	0.0009
FH	0.059	0.40
Clusterin	–0.08	0.26
FI	0.13	0.07
TCC	0.17	0.02
iC3b	–0.059	0.40
Bb	–0.013	0.85
C4d	0.11	0.13

measured at a plasma dilution of 1:300. There was no detectable inter-assay interference between the different analytes in the multiplex and intra- and inter-assay, confirming the suitability of the assay sets chosen for multiplexing. CVs were < 10% for all analytes in the multiplex (data not shown).

C9, FI and TCC levels correlate with age

Correlation with donor age at sampling was tested for all complement analytes in the complete set of samples (Table 2). C9 levels showed a strong positive correlation with donor age at time of sampling. Levels of FI and TCC demonstrated weak but significant positive correlations with donor age at time of sampling. Other complement analytes did not significantly correlate with donor age.

Clusterin is the sole plasma complement biomarker that distinguishes AD from control

Of the nine complement analytes measured (FH variants combined to give total FH), only one, clusterin, was significantly different between AD and control populations (Table 3). The mean plasma clusterin concentration in controls was 295 mg/l and in AD was 388 mg/l, a highly significant difference ($p = 2.32 \times 10^{-6}$). A model combining clusterin with co-variables associated with AD (APOE status and age) was highly predictive with an AUC of 0.66 for the test set and 0.78 for the entire sample set (Table 3; Fig. 1A). At 70% sensitivity, the predicted specificity of the model was 75%.

Three complement analytes differentiate MCI convertors from non-convertors

From the analysis of MCI convertors versus non-convertors, three of the nine complement analytes were significantly different between the groups: clusterin, TCC, and FI (Table 4; Fig. 1B). Of these, clusterin was the most significant; the mean clusterin level in non-convertors was 309 mg/l and in convertors was 418 mg/l. TCC was significantly lower in MCI convertors compared to non-convertors (0.7 mg/l versus 3.6 mg/l), while FI was significantly reduced in MCI convertors compared to non-convertors (27.7 mg/l versus 50.7 mg/l; the latter identical to healthy controls). From these data, a model was constructed combining clusterin, TCC, and FI with the sole co-variable associated with MCI conversion (APOE status); the model was highly predictive of conversion with an AUC of 0.85 for the entire sample set (Table 4, Fig. 1B). At 80% sensitivity, the predicted specificity of the model was 79%.

Measurement of FH Y402 H allotypic variants predicts progression in MCI

In the selected model, total FH concentration was not significantly reduced in AD compared to controls (335.3 mg/l versus 350.8 mg/l; Table 3)

Table 3
Mixed effects linear model for complement analyte difference between AD and controls. Clustered mixed-effects linear modeling (using the lme4 and lmerTest R packages) was used to explore the associations between each variable and disease status. The variables which were most strongly associated with diagnosis (based on *p* value) were then combined into one model (final model). Any variables which were not significant after inclusion in the model were discarded. Note that final model for AD versus controls comprises Clusterin, APOE4, and age

Initial Model	AD (mean ± SD; mg/l)	Controls (mean ± SD; mg/l)	β (95% CI)	*p*
C1s	102.2 ± 19.4	104.1 ± 20.4	–0.001 (–0.005 – 0.003)	0.54
C4d	3.8 ± 4.5	2.9 ± 6.2	0.014 (–0.017 – 0.05)	0.39
C9	52.0 ± 17.1	51.2 ± 14.8	0.001 (–0.003 – 0.006)	0.65
Clusterin	387.6 ± 113.9	295.0 ± 128.5	–0.001 (–0.002 – –0.0004)	$\mathbf{2.32 \times 10^{-6}}$
FI	51.5 ± 37.8	50.7 ± 38.9	0.001 (–0.002 – 0.004)	0.32
TCC	3.2 ± 4.3	2.8 ± 2.3	–0.016 (–0.043 – 0.011)	0.27
iC3b	1.8 ± 1.2	1.6 ± 1.1	0.003 (–0.062 – 0.068)	0.93
Bb	21.2 ± 9.3	18.7 ± 8.8	0.003 (–0.005 – 0.010)	0.51
FH	335.3 ± 81.0	350.8 ± 99.0	0.0004 (–0.0004 – 0.001)	0.34
Gender (male)			–0.082 (–0.21 – 0.05)	0.23
APOE4			–0.12 (–0.23 – –0.008)	0.039
Age at sample			–0.021 (–0.03 – –0.01)	$\mathbf{4.75 \times 10^{-5}}$
Final model AD versus control			β (95% CI)	*p*
Clusterin			–0.001 (–0.002 – –0.0008)	8.1×10^{-7}
APOE4			–0.13 (–0.2378 – –0.02)	0.02
Age at sample			–0.02 (–0.03 – –0.01)	2.4×10^{-5}

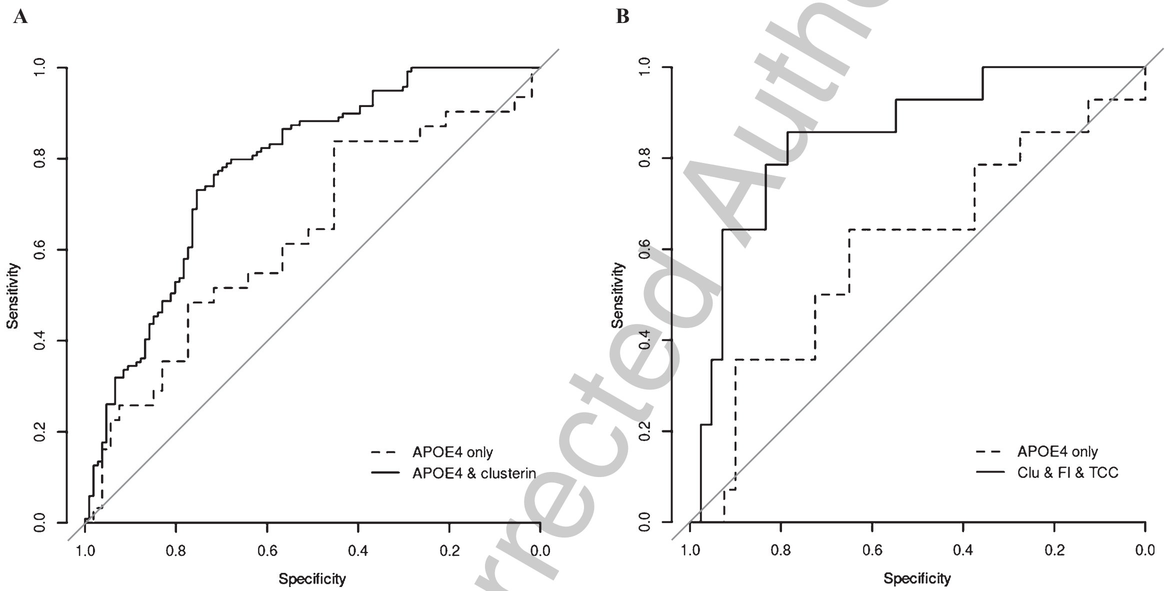

Fig. 1. Receiver-operating characteristic (ROC) curves representing models which differentiate AD from controls (A) and MCI convertors from non-convertors (B). ROC curves were drawn for the final models distinguishing AD from controls (A; clusterin and APOE4) and MCI convertors from non-convertors (B; clusterin, FI, and TCC). The area under the curve (AUC) for the final model was calculated, and compared to that for APOE4 alone. AUC was used to define the predictive power of the analyte or analyte set that comprised the model; the predictive power of the model for distinguishing AD from controls was 0.78, and for predicting conversion was 0.85.

or in MCI convertors compared to non-convertors (297.9 mg/l versus 351.4 mg/l; Table 4). When the levels of the Y402 and H402 variants of FH, measured separately using variant-specific capture antibodies and corrected for allele number, were compared between AD and control groups, there was no significant difference (Y variant, AD, 170.4 mg/l, controls, 172.5 mg/l; H variant, AD, 167 mg/l, controls, 175.5 mg/l; Table 5). A similar analysis comparing MCI mon-convertors and convertors revealed a significantly lower level of the H variant in the convertors (Y variant, non-convertors, 166.3 mg/l. convertors, 164.4 mg/l; H variant, non-convertors, 172.5 mg/l, convertors, 142.1 mg/l; Table 5; $p = 0.0056$).

Table 4
Mixed effects linear model for complement analyte difference between MCI convertors and non-convertors. Clustered mixed-effects linear modeling (using the lme4 and lmerTest R packages) was used to explore the associations between each variable and disease status. The variables which were most strongly associated with diagnosis (based on *p* value) were then combined into one model (final model). Any variables which were not significant after inclusion in the model were discarded. Note that final model for conversion versus non-conversion comprises Clusterin, FI, and TCC

Initial Model	Converted to AD (mean ± SD; mg/l)	Not converted (mean ± SD; mg/l)	β (95% CI)	*p*
C1s	88.9 ± 15.1	103.2 ± 24.8	–0.003 (–0.006– 0.0009)	0.15
C4d	2.2 ± 2.1	3.6 ± 3.2	–0.017 (–0.042– 0.008)	0.18
C9	42.8 ± 16.6	50.5 ± 14.1	0.002 (–0.0027 – 0.006)	0.43
Clusterin	417.5 ± 88.5	308.7 ± 115.2	0.002 (0.001 – 0.002)	**2.43×10^{-7}**
FI	27.7 ± 7.9	50.7 ± 26.6	–0.006 (–0.009 – –0.002)	**0.0025**
TCC	0.7 ± 2.5	3.6 ± 3.4	–0.03 (–0.04 – –0.01)	**0.0027**
iC3b	1.7 ± 1.2	1.7 ± 0.9	0.02 (–0.05 – 0.08)	0.67
Bb	18.3 ± 9.3	18.4 ± 8.8	0.003 (–0.005 – 0.01)	0.51
FH	297.9 ± 75	351.4 ± 96.8	–0.0001 (–0.001 – 0.0008)	0.76
Gender (male)			0.11 (–0.02 – 0.24)	0.10
APOE-ε4			–0.02 (–0.11 – 0.08)	0.71
Age at sample			0.0004 (–0.009 – 0.01)	0.94
Final model convertor versus non-convertor			β (95% CI)	p
Clusterin			0.002 (0.001 – 0.002)	1.26×10^{-9}
FI			–0.006 (–0.009 – –0.003)	1.42×10^{-5}
TCC			–0.024 (–0.04 – –0.008)	0.005

Table 5
FH allotypes in AD and MCI. In an initial analysis there was no association between FH-Y402H genotype or diagnosis and FH plasma levels, but plasma FH levels did predict whether patients convert from MCI to AD (by ANOVA, $p = 0.00330$. Allele number-corrected allotype levels in FH-Y402H heterozygotes were then compared between AD and controls (top) and MCI non convertors and convertors (bottom); FH-H402 levels were significantly lower (**) in MCI patients who subsequently converted to AD when compared to those who did not convert

FH allotype levels in AD and controls (mean ± SD; mg/l)			
diagnosis	Y402	H402	*p*
AD	170.4 ± 45.1	167.0 ± 39.8	0.71
control	172.5 ± 42.7	175.5 ± 38.2	0.71
p	0.81	0.26	
FH allotype levels in MCI convertors and non-convertors (mean ± SD; mg/l)			
converted	Y402	H402	*p*
no	166.3 ± 47.2	172.5 ± 41.6	0.44
yes	164.4 ± 57.3	142.1 ± 34.5	0.18
p	0.90	0.0056**	

DISCUSSION

A plasma marker or marker set that is indicative of pathology or predictive of conversion to AD in individuals with MCI, or disease course in patients with early AD is sorely needed to facilitate early diagnosis and inform selection of participants into future clinical trials, particularly those targeting immune system involvement and inflammation. The abundant evidence implicating inflammation, and specifically complement, in pathogenesis led us to explore the complement system as a source of biomarkers. Guided by literature evidence and reagent availability, we selected ten complement analytes and designed a multiplex assay to measure all simultaneously. Our data demonstrate that clusterin alone among the analytes tested significantly differentiated AD patients from matched controls, while clusterin, FI, and TCC were all significantly different between individuals with MCI who subsequently either converted to AD or remained stable when reassessed at one year post-sampling. In our sample set, 26% of the MCI cases progressed to dementia at one year; this is markedly higher than published annual conversion rates, typically around 10%, although considerable variation between sample sets has been noted [27].

Several published studies have reported elevated plasma levels of clusterin in AD compared to controls in diverse ethnic groups [17–20, 28–30]. Taken together with our findings, these data demonstrate that elevated plasma clusterin level is a robust marker for AD that is replicated across different assay platforms. In light of this, it is somewhat paradoxical that two disease-associated SNP in clusterin are reported to associate with decreased plasma levels [29, 30]. Precisely how plasma clusterin levels impact disease risk remains uncertain. Clusterin is a multifunctional molecule, an inhibitor of the complement terminal pathway but also a professional molecular chaperone involved in clearance of debris [31]. Amyloid plaques in AD are richly decorated with clusterin and a role in clearance of amyloid has been proposed [32, 33]. Clusterin has also been shown to reduce $A\beta_{42}$ toxicity in a rat model of AD [34].

Association of plasma clusterin levels with rate of cognitive decline has been reported both in MCI and AD [17, 20, 24]; in each of these studies, higher clusterin levels predicted more rapid decline. Our data demonstrating substantially higher plasma clusterin in MCI donors who subsequently convert to AD compared to non-convertors robustly support these findings and show that elevated plasma clusterin is a powerful predictor of progression. The functional basis of this association is problematic; if clusterin is involved in reducing Aβ toxicity and accelerating amyloid clearance, then increased plasma levels might be expected to restrict the development of pathology. It is possible that increased clusterin production, reflected in increased plasma levels, represents a failed protective response to the disease process.

TCC, a marker of complement terminal pathway activation, is present on neurons, plaques, and adjacent blood vessels in AD brain [32–35]; fluid-phase TCC (also termed sC5b-9) has been measured in AD CSF [36], but plasma levels of TCC have not previously been reported in AD or MCI. Plasma TCC levels were not different between AD and controls but were significantly lower in MCI donors who subsequently converted to AD compared to non-convertors; this finding is somewhat counterintuitive in that it implies lower levels of terminal pathway activation in the convertors despite clear evidence that the terminal pathway is abundantly activated in AD brain. We suggest that the demonstrated elevated levels of clusterin, an efficient inhibitor of the terminal pathway, in the convertor group might suppress terminal pathway activation and TCC generation in plasma. Notably, plasma levels of activation pathway products (iC3b, C4d, Bb) were not different between the groups, suggesting that any difference in central complement activation between MCI convertors and non-convertors was not reflected in the periphery.

FI is the enzyme responsible for regulating the activation pathway convertases; complement receptor 1, linked through GWAS studies to AD [9, 37, 38], is the major cell-associated cofactor for FI-mediated cleavage of C3b/C4b. Plasma FI has previously been reported as a biomarker of brain atrophy [22]. Here we show that plasma FI level was significantly reduced in MCI convertors compared to non-convertors (27.7 mg/l versus 50.7 mg/l; the latter identical to healthy controls). Lower levels of FI will impact capacity to control complement activation once triggered and favor dysregulation [39].

In the mixed effects linear models described here, FH was not significantly different between AD patients compared to controls (335.3 mg/l versus 350. 8 mg/l; Table 3) or between MCI convertors compared to non-convertors (297.9 mg/l versus 351. 4 mg/l; Table 4). The common FH-Y402 H polymorphism is a major risk factor for AMD [12, 13], but does not associate with AD in multiple studies [14–16]. Here we separately measured plasma levels of the products of the two allotypic variants and showed that levels of the FH-H402 variant were significantly lower in MCI donors who subsequently converted to AD compared to non-convertors. These data could be explained by decreased expression of the FH-H402 allele in the convertor group or, more likely, by increased consumption of the FH- H402 allotype protein in response to the disease in MCI convertors. Differential binding of the different FH-Y402 H allotypes at sites of pathology has previously been described in the context of AMD [40], and our unpublished data suggest preferential binding of the FH-H402 allotype in AD brain.

From our data we generated two models. The first compared AD patients with controls and included clusterin with the co-variables age and APOE status; ROC curves constructed from this set gave an AUC of 0.78 for the entire set, considered "moderately predictive" [41]. The second model compared MCI convertors and non-convertors and included clusterin, TCC, and FI with the sole co-variable associated with MCI conversion (APOE status); ROC curves gave an AUC of 0.85 for the entire sample set, considered "highly predictive". Although levels of the FH-H402 allotype were significantly predictive of MCI conversion when measured in FH-Y402 H heterozygotes, this variable was not included in the model because it applied only to the subset of the population that possessed one or more H402 alleles; nevertheless, the data demonstrate that measuring plasma allotype levels for a common complement polymorphism can help predict disease and raise the prospect that other complement polymorphisms might also be predictive, as is the case in AMD [12, 13].

In summary, we show that combinations of complement biomarkers can aid diagnosis and prediction of outcome in MCI and AD. The results described were from an initial set of just ten complement analytes and from these only one was predictive for distinguishing AD from controls and three for predicting progression in MCI. Expanding the test set of complement biomarkers will add other predictive analytes that will strengthen the predictive power of the marker set and provide further information on precisely how complement contributes to AD pathology. Adding in non-complement biomarkers will likely further strengthen and contribute to an optimum multiplex for diagnosis and prediction of outcome. The demonstration that complement activation occurs in MCI and predicts conversion strengthens the case for testing anti-complement therapies in this group.

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REFERENCES

[1] Hye A, Lynham S, Thambisetty M, Causevic M, Campbell J, Byers HL, Hooper C, Rijsdijk F, Tabrizi SJ, Banner S, Shaw CE, Foy C, Poppe M, Archer N, Hamilton G, Powell J, Brown RG, Sham P, Ward M, Lovestone S (2006) Proteome-based plasma biomarkers for Alzheimer's disease. *Brain* **129**, 3042-3050.

[2] Song F, Poljak A, Smythe GA, Sachdev P (2009) Plasma biomarkers for mild cognitive impairment and Alzheimer's disease. *Brain Res Rev* **61**, 69-80.

[3] Zabel M, Schrag M, Mueller C, Zhou W, Crofton A, Petersen F, Dickson A, Kirsch WM (2012) Assessing candidate serum biomarkers for Alzheimer's disease: A longitudinal study. *J Alzheimers Dis* **30**, 311-321.

[4] Snyder HM, Carrillo MC, Grodstein F, Henriksen K, Jeromin A, Lovestone S, Mielke MM, O'Bryant S, Sarasa M, Sjøgren M, Soares H, Teeling J, Trushina E, Ward M, West T, Bain LJ, Shineman DW, Weiner M, Fillit HM (2014) Developing novel blood-based biomarkers for Alzheimer's disease. *Alzheimers Dement* **10**, 109-114.

[5] Sattlecker M, Kiddle SJ, Newhouse S, Proitsi P, Nelson S, Williams S, Johnston C, Killick R, Simmons A, Westman E, Hodges A, Soininen H, Kłoszewska I, Mecocci P, Tsolaki M, Vellas B, Lovestone S, AddNeuroMed Consortium, Dobson RJ (2014) Alzheimer's disease biomarker discovery using SOMAscan multiplexed protein technology. *Alzheimers Dement* **10**, 724-734.

[6] Heppner FL, Ransohoff RM, Becher B (2015) Immune attack: The role of inflammation in Alzheimer disease. *Nat Rev Neurosci* **16**, 358-372.

[7] Julian A, Dugast E, Ragot S, Krolak-Salmon P, Berrut G, Dantoine T, Hommet C, Hanon O, Page G, Paccalin M (2015) There is no correlation between peripheral inflammation and cognitive status at diagnosis in Alzheimer's disease. *Aging Clin Exp Res* **27**, 589-594.

[8] Yarchoan M, Louneva N, Xie SX, Swenson FJ, Hu W, Soares H, Trojanowski JQ, Lee VM, Kling MA, Shaw LM, Chen-Plotkin A, Wolk DA, Arnold SE (2013) Association of plasma C-reactive protein levels with the diagnosis of Alzheimer's disease. *J Neurol Sci* **333**, 9-12.

[9] Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, DeStafano AL, Bis JC, Beecham GW, Grenier-Boley B, Russo G, Thorton-Wells TA, Jones N, Smith AV, Chouraki V, Thomas C, Ikram MA, Zelenika D, Vardarajan BN, Kamatani Y, Lin CF, Gerrish A, Schmidt H, Kunkle B, Dunstan ML, Ruiz A, Bihoreau MT, Choi SH, Reitz C, Pasquier F, Cruchaga C, Craig D, Amin N, Berr C, Lopez OL, De Jager PL, Deramecourt V, Johnston JA, Evans D, Lovestone S, Letenneur L, Morón FJ, Rubinsztein DC, Eiriksdottir G, Sleegers K, Goate AM, Fiévet N, Huentelman MW, Gill M, Brown K, Kamboh MI, Keller L, Barberger-Gateau P, McGuiness B, Larson EB, Green R, Myers AJ, Dufouil C, Todd S, Wallon D, Love S, Rogaeva E, Gallacher J, St George-Hyslop P, Clarimon J, Lleo A, Bayer A, Tsuang DW, Yu L, Tsolaki M, Bossù P, Spalletta G, Proitsi P, Collinge J, Sorbi S, Sanchez-Garcia F, Fox NC, Hardy J, Deniz Naranjo MC, Bosco P, Clarke R, Brayne C, Galimberti D, Mancuso M, Matthews F, European Alzheimer's Disease Initiative (EADI), Genetic, Environmental Risk in Alzheimer's Disease, Alzheimer's Disease Genetic Consortium, Cohorts for Heart and Aging Research in Genomic Epidemiology, Moebus S, Mecocci P, Del Zompo M, Maier W, Hampel H, Pilotto A, Bullido M, Panza F, Caffarra P, Nacmias B, Gilbert JR, Mayhaus M, Lannefelt L, Hakonarson H, Pichler S, Carrasquillo MM, Ingelsson M, Beekly D, Alvarez V, Zou F, Valladares O, Younkin SG, Coto E, Hamilton-Nelson KL, Gu W, Razquin C, Pastor P, Mateo I, Owen MJ, Faber KM, Jonsson PV, Combarros O, O'Donovan MC, Cantwell LB, Soininen H, Blacker D, Mead S, Mosley TH Jr, Bennett DA, Harris TB, Fratiglioni L, Holmes C, de Bruijn RF, Passmore P, Montine TJ, Bettens K, Rotter JI, Brice A, Morgan K, Foroud TM, Kukull WA, Hannequin D, Powell JF, Nalls MA, Ritchie K, Lunetta KL, Kauwe JS, Boerwinkle E, Riemenschneider M, Boada M, Hiltuenen M, Martin ER, Schmidt R, Rujescu D, Wang LS, Dartigues JF, Mayeux R, Tzourio C, Hofman A, Nöthen MM, Graff C, Psaty BM, Jones L, Haines JL, Holmans PA, Lathrop M, Pericak-Vance MA, Launer LJ, Farrer LA, van Duijn CM, Van Broeckhoven C, Moskvina V, Seshadri S, Williams J, Schellenberg GD, Amouyel P (2013) Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat Genet* **45**, 1452-1458.

[10] Emmerling MR, Watson MD, Raby CA, Spiegel K (2000) The role of complement in Alzheimer's disease pathology. *Biochim Biophys Acta* **1502**, 158-171.

[11] Fonseca MI, Chu SH, Berci AM, Benoit ME, Peters DG, Kimura Y, Tenner AJ (2011) Contribution of complement activation pathways to neuropathology differs among mouse models of Alzheimer's disease. *J Neuroinflammation* **8**, 4.

[12] Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R (2005) A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* **102**, 7227-7232.

[13] Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J (2005) Complement factor H polymorphism in age-related macular degeneration. *Science* **308**, 385-389.

[14] Williams MA, Haughton D, Stevenson M, Craig D, Passmore AP, Silvestri G (2015) Plasma complement factor H in Alzheimer's disease. *J Alzheimers Dis* **45**, 369-372.

[15] Le Fur I, Laumet G, Richard F, Fievet N, Berr C, Rouaud O, Delcourt C, Amouyel P, Lambert JC (2010) Association study of the CFH Y402H polymorphism with Alzheimer's disease. *Neurobiol Aging* **31**, 165-166.

[16] Hamilton G, Proitsi P, Williams J, O'Donovan M, Owen M, Powell J, Lovestone S (2007) Complement factor H Y402H polymorphism is not associated with late-onset Alzheimer's disease. *Neuromolecular Med* **9**, 331-334.

[17] Thambisetty M, Simmons A, Velayudhan L, Hye A, Campbell J, Zhang Y, Wahlund LO, Westman E, Kinsey A, Güntert A, Proitsi P, Powell J, Causevic M, Killick R, Lunnon K, Lynham S, Broadstock M, Choudhry F, Howlett DR, Williams RJ, Sharp SI, Mitchelmore C, Tunnard C, Leung R, Foy C, O'Brien D, Breen G, Furney SJ, Ward M, Kloszewska I, Mecocci P, Soininen H, Tsolaki M, Vellas B, Hodges A, Murphy DG, Parkins S, Richardson JC, Resnick SM, Ferrucci L, Wong DF, Zhou Y, Muehlboeck S, Evans A, Francis PT, Spenger C, Lovestone S (2010) Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. *Arch Gen Psychiatry* **67**, 739-748.

[18] Schrijvers EM, Koudstaal PJ, Hofman A, Breteler MM (2011) Plasma clusterin and the risk of Alzheimer disease. *JAMA* **305**, 1322-1326.

[19] Thambisetty M, An Y, Kinsey A, Koka D, Saleem M, Güntert A, Kraut M, Ferrucci L, Davatzikos C, Lovestone S, Resnick SM (2012) Plasma clusterin concentration is associated with longitudinal brain atrophy in mild cognitive impairment. *Neuroimage* **59**, 212-217.

[20] Jongbloed W, van Dijk KD, Mulder SD, van de Berg WD, Blankenstein MA, van der Flier W, Veerhuis R (2015) Clusterin Levels in plasma predict cognitive decline and progression to Alzheimer's disease. *J Alzheimers Dis* **46**, 1103-1110.

[21] IJsselstijn L, Dekker LJ, Koudstaal PJ, Hofman A, Sillevis Smitt PA, Breteler MM, Luider TM (2011) Serum clusterin levels are not increased in presymptomatic Alzheimer's disease. *J Proteome Res* **10**, 2006-2010.

[22] Thambisetty M, Simmons A, Hye A, Campbell J, Westman E, Zhang Y, Wahlund LO, Kinsey A, Causevic M, Killick R, Kloszewska I, Mecocci P, Soininen H, Tsolaki M, Vellas B, Spenger C, Lovestone S; AddNeuroMed Consortium (2011) Plasma biomarkers of brain atrophy in Alzheimer's disease. *PLoS One* **6**, e28527.

[23] International Genomics of Alzheimer's Disease Consortium (IGAP) (2015) Convergent genetic and expression data implicate immunity in Alzheimer's disease. *Alzheimers Dement* **11**, 658-671.

[24] Hye A, Riddoch-Contreras J, Baird AL, Ashton NJ, Bazenet C, Leung R, Westman E, Simmons A, Dobson R, Sattlecker M, Lupton M, Lunnon K, Keohane A, Ward M, Pike I, Zucht HD, Pepin D, Zheng W, Tunnicliffe A, Richardson J, Gauthier S, Soininen H, Kłoszewska I, Mecocci P, Tsolaki M, Vellas B, Lovestone S (2014) Plasma proteins predict conversion to dementia from prodromal disease. *Alzheimers Dement* **10**, 799-807.

[25] Leung R, Proitsi P, Simmons A, Lunnon K, Guntert A, Kronenberg D, Pritchard M, Tsolaki M, Mecocci P, Kloszewska I, Vellas B, Soininen H, Wahlund LO, Lovestone S (2013) Inflammatory proteins in plasma are associated with severity of Alzheimer's disease. *PLoS One* **8**, e64971.

[26] Hakobyan S, Tortajada A, Harris CL, de Córdoba SR, Morgan BP (2010) Variant-specific quantification of factor H in plasma identifies null alleles associated with atypical hemolytic uremic syndrome. *Kidney Int* **78**, 782-788.

[27] Mitchell AJ, Shiri-Feshki M (2009) Rate of progression of mild cognitive impairment to dementia – meta-analysis of 41 robust inception cohort studies. *Acta Psychiatr Scand* **119**, 252-265.

[28] Schürmann B, Wiese B, Bickel H, Weyerer S, Riedel-Heller SG, Pentzek M, Bachmann C, Williams J, van den Bussche H, Maier W, Jessen F (2013) Association of the Alzheimer's disease clusterin risk allele with plasma clusterin concentration. *J Alzheimers Dis* **25**, 421-424.

[29] Mullan GM, McEneny J, Fuchs M, McMaster C, Todd S, McGuinness B, Henry M, Passmore AP, Young IS, Johnston JA (2013) Plasma clusterin levels and the rs11136000 genotype in individuals with mild cognitive impairment and Alzheimer's disease. *Curr Alzheimer Res* **10**, 973-978.

[30] Xing YY, Yu JT, Cui WZ, Zhong XL, Wu ZC, Zhang Q, Tan L (2012) Blood clusterin levels, rs9331888 polymorphism, and the risk of Alzheimer's disease. *J Alzheimers Dis* **29**, 515-519.

[31] Jenne DE, Tschopp J (1992) Clusterin: The intriguing guises of a widely expressed glycoprotein. *Trends Biochem Sci* **17**, 154-159.

[32] Calero M, Rostagno A, Matsubara E, Zlokovic B, Frangione B, Ghiso J (2000) Apolipoprotein J (clusterin) and Alzheimer's disease. *Microsc Res Tech* **50**, 305-315.

[33] Almeida MR, Saraiva MJ (2012) Clearance of extracellular misfolded proteins in systemic amyloidosis: Experience with transthyretin. *FEBS Lett* **586**, 2891-2896.

[34] Cascella R, Conti S, Tatini F, Evangelisti E, Scartabelli T, Casamenti F, Wilson MR, Chiti F, Cecchi C (2013) Extracellular chaperones prevent Aβ42-induced toxicity in rat brains. *Biochim Biophys Acta* **1832**, 1217-1226.

[35] Itagaki S, Akiyama H, Saito H, McGeer PL (1994) Ultrastructural localization of complement membrane attack complex (MAC)-like immunoreactivity in brains of patients with Alzheimer's disease. *Brain Res* **645**, 78-84.

[36] Webster S, Lue LF, Brachova L, Tenner AJ, McGeer PL, Terai K, Walker DG, Bradt B, Cooper NR, Rogers J (1997) Molecular and cellular characterization of the membrane attack complex, C5b-9, in Alzheimer's disease. *Neurobiol Aging* **18**, 415-421.

[37] D'Andrea MR (2005) Evidence that immunoglobulin-positive neurons in Alzheimer's disease are dying via the classical antibody-dependent complement pathway. *Am J Alzheimers Dis Other Demen* **20**, 144-150.

[38] Luo J, Li S, Qin X, Song L, Peng Q, Chen S, Xie Y, Xie L, Li T, He Y, Deng Y, Wang J, Zeng Z (2014) Meta-analysis of the association between CR1 polymorphisms and risk of late-onset Alzheimer's disease. *Neurosci Lett* **578**, 165-170.

[39] Lay E, Nutland S, Smith JE, Hiles I, Smith RA, Seilly DJ, Buchberger A, Schwaeble W, Lachmann PJ (2015) Complotype affects the extent of down-regulation by Factor I of the C3b feedback cycle *in vitro*. *Clin Exp Immunol* **181**, 314-322.

[40] Clark SJ, Perveen R, Hakobyan S, Morgan BP, Sim RB, Bishop PN, Day AJ (2010) Impaired binding of the age-related macular degeneration-associated complement factor H 402H allotype to Bruch's membrane in human retina. *J Biol Chem* **285**, 30192-30202.

[41] Mandic S, Go C, Aggarwal I, Myers J, Froelicher VF (2008) Relationship of predictive modeling to receiver operating characteristics. *J Cardiopulm Rehabil Prev* **28**, 415-419.